Novel Therapeutic Intervention for Myocardial infarction in large animal Model

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Thank you to Juan L. Luis, Juan F. Gil-Mercedes, Juan Mercedes, Francis Gil, Melody (my good friend), and Kelsey Speer for their support and for everything.

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List of Abbreviations

AAV9: Adeno-Associated Virus serotype 9
BSA: Bovine serum albumin
CMV: Cytomegalovirus
GFP: Green Fluorescent Protein
GFP+: GFP-adenovirus transduced cells
GMTMM: transcription factors for cardiomyocyte reprogramming
- G: Gata4
- M: Mef2c
- T: Tbx5
- M: Mesp1
- M: Myocardin

LVEDV: Left ventricle End-Diastolic Volume
LVESD: Left ventricle End-Systolic Volume
LV: Left ventricle
Min: minute
Vim: Vimentin

Abstract

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Maria Mercedes

John Gearhart, PhD

Heart failure (HF) represents an enormous clinical problem that imposes a significant burden both to society and survivors. During a myocardial infarction, cardiomyocytes are damaged due to a lack of oxygen to the left ventricle and as a result these cells die. The dead cells are then replaced by fibrotic scar tissue that inhibits the heart from properly pumping blood to the rest of the heart. The current treatments of HF do not reverse the damage that the left ventricle experiences and as a result many people experience recurrent heart failure and sometime cardiac arrest. The field of cellular reprogramming investigates the process of direct reprogramming of endogenous cardiac fibroblasts to cardiomyocytes as a novel approach to help the heart to pump blood properly after a myocardial infarction. In this project, we used a biodegradable hyaluronic acid (HA) hydrogel to encapsulate Adeno-associated virus (AAV) 9 engineered to deliver the reporter gene green fluorescent protein (GFP) or five transcription factors (G: Gata4, M: Mef2c, T: Tbx5, M: Mesp1and M: Myocardin) in pigs' heart post-myocardial. Our method represents a unique approach to transfect cardiomyocyte cells *in vivo* post-myocardial infarct in pig. This study also demonstrates a cardiac functional improvement with the injection of hydrogel gel, AAV9, and selected transcription factors in the compromised area of the left ventricle. This novel method reduced the scar tissue in the compromised area of the heart compared to control.

1. BACKGROUND

Heart failure (HF), according to the Center for Disease Control and Prevention (CDC) and the American Heart Association (AHA), imposes a great financial burden to the United States health care systems (Benjamin et al., 2017). The AHA predicts that by 2030, every U.S. taxpayer will pay more in taxes to care for HF; this means that every U.S. citizen will pay more in the cost of treating HF. The lifetime cost of HF care is very high primarily due to hospitalization. Currently, more Medicare dollars are expended on treatment of HF than on any other disease. Since HF is primarily a disease of the elderly, its prevalence will increase as the population ages, as shown on Fig. 1 (Heidenreich et al., 2013). Along with the financial burden, this ailment decreases the quality of life of the survivors. These patients experience various emotional and physical impairment like fatigue, depression, chest pain, and sleeping difficulties and, as a result, mortality increases in this population as well (Dunlay et al., 2011).

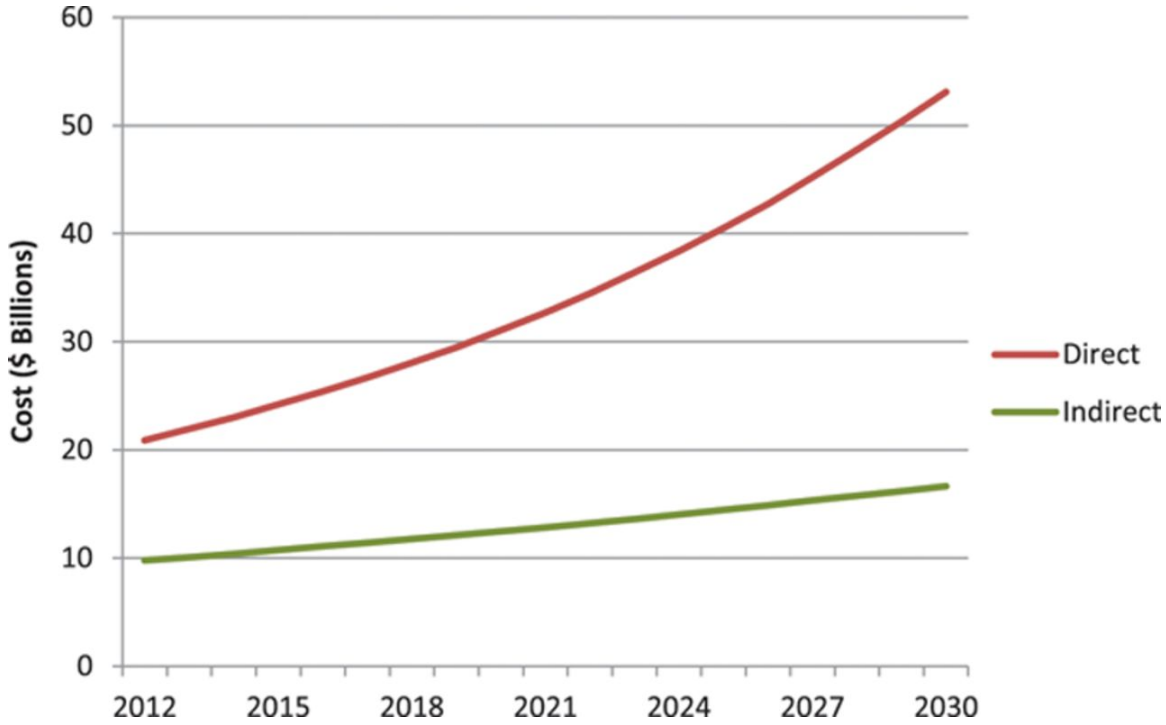

Figure 1. Image adapted from Heidenreich et al., 2013. This graph shows the increase in direct and indirect costs attributable to HF from 2012 to 2030.

HF is primarily caused by coronary artery disease (CAD), myocardial infarction (MI), and cardiomyopathy; these three myocardial dysfunctions result in the death of cells that compose the cardiac muscle (i.e. myocardial cell death). Since the adult mammalian heart does not regenerate, it cannot repopulate lost cardiomyocytes after HF (Li & Izpisua Belmonte, 2016). The left ventricle (LV) is the chamber most affected in HF and within it approximately 2 to 4 billion cardiomyocytes that are damaged and replaced by fibrotic scar tissue after a myocardial infarction (Laflamme & Murry, 2011). This maladaptive restorative process causes the infarcted area to electrically uncouple from the rest of the myocardium, and the loss of contractile function leads to recurrent heart failure (Li & Izpisua Belmonte, 2016). Current treatments for this illness are pharmacologic

intervention, changes in lifestyle, transplantation (which is very limited), cardiac device therapy, and palliative care (Lemond & Allen, 2011). The caveat with all of the above interventions is that they do not fully restore heart function. Thus, it is imperative to develop a new therapeutic treatment for cardiac repair.

In order to tackle this public health problem, scientists have been developing different approaches to better treat MI, as shown in Figure 2 (Li & Izpisua Belmonte, 2016). One of these approaches is based upon the well accepted Nobel Prize winning experiments (i.e., induced pluripotent stem cells (iPSCs) which holds great promise but has concerning caveats. iPSCs are generated by forcing the expression of exogenous genes in somatic cells (i.e. Yamanaka factors) (Takahashi & Yamanaka, 2006). These induced cells can give rise to all the cells of the body, but there is a possibility that these cells may form tumors. Important to our study, iPSCs have been differentiated into cardiomyocytes and then transplanted into an ischemic heart. iPSC-derived cardiomyocytes are used to treat MI in mice (Rojas et al., 2017). Another challenge that these approaches face is that the induced cells may not electrically couple and synchronize with the host myocardium. Furthermore, after a decade of research with iPSCs, the mechanism of how the Yamanaka factor induces iPSCs is still unknown (Scudellari, 2016).

Other therapeutic approaches include the use of fetal and adult stem cells to give rise to cardiomyocytes (Boyle, Schulman, Hare, & Oettgen, 2006). These alternatives have not been proven successful because the induced cardiomyocytes are fully differentiated into mature cardiomyocytes. For instance, in a stem cell experiment on non-human primate hearts, (Chong et al., 2014) demonstrated that the arrhythmic complications occur when

human embryonic-stem-cell-derived cardiomyoctyes were used to treat MI. However, the group believe that arrhythmias could be managed by continuous surveillance and proper care of the animals (Murry, Chong, & Laflamme, 2014). Despite the potential of these cells to address HF, stem cells can also develop into teratomas. Hence, further study is important to develop novel therapeutic modalities to treat and perhaps cure cardiac failure.

Another approach investigated in the field of reprogramming is direct lineage conversion, to change a terminally differentiated cell into another, *in vivo* (Fig. 2). This methodology relies upon the injection of transcription factors known to be involved in cardiomyocytes development and function directly into the compromised area of the heart post-MI. The target cells are cardiac fibroblasts, since these cells are abundant in the heart and have been successfully reprogrammed by many groups in the field. A limitation of this approach is that the virus used to transfect cardiomyocytes diffuses in the heart tissue. As a result, the transfected cells are not found in the site of injection. Finding the transfected cells far away from the compromised area is a problem because the induced cells are required in the compromised area of the heart.

In summary, HF is a chronic illness that imposes a burden both to society and patients. The costs of HF treatment to society are substantial. For patients, the burden is also significant because many of them experience physical limitations and psychological effects such as anxiety and depression. In order to address this illness, better approaches need to be developed to treat this ailment.

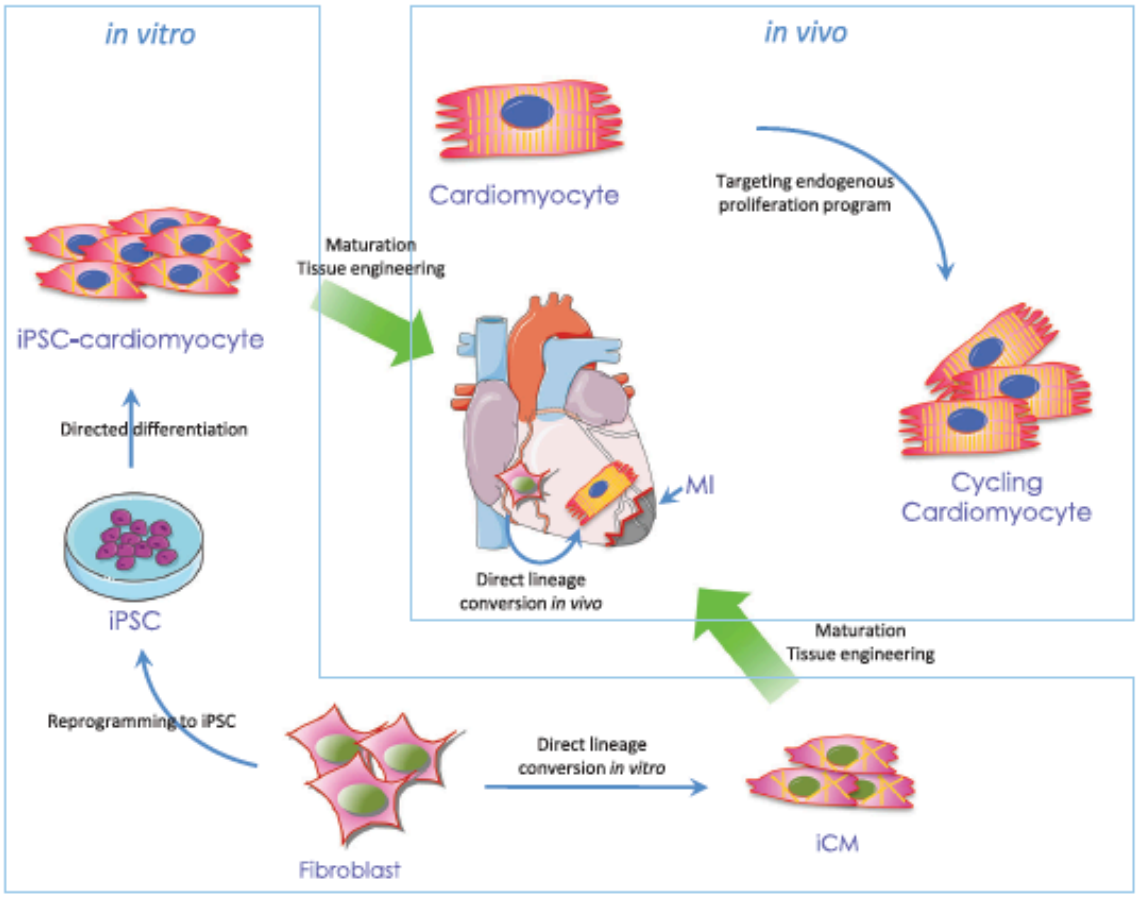

Figure 2. Image adapted from Li et al., 2016. Studies of different therapeutic approaches attempting to replace loss cardiomyocytes after myocardial infarction.

THESIS OBJECTIVE

Our laboratory wanted to know whether the AAV9 virus was an effective means of supplying the transcription factors to heart tissue for lineage conversion of fibroblasts to myocardial cells. Preliminary work from our laboratory demonstrated that injecting Adeno-associated virus (AAV) 9 engineered to deliver the reporter gene GFP in pig heart post-myocardial infarction was successful but diffuses in the compromised left ventricle. After serially sectioning the heart tissue where the virus was injected, the transfected cells were detected by immunohistochemistry far away from the point of injection. Since biodegradable hyaluronic acid (HA) hydrogel are known to be great delivery vehicles, we decided to use HA to encapsulate the virus to transfect cardiomyocytes at the site of injection. Hence, the objective of this thesis work was to determine if hydrogel maintain AVV9 virus at the point of injection and restrict diffusion of the virus and improve transfected cardiomyocytes post-MI in pigs.

Specific Aim 1: To determine the ability of hydrogel to restrict AVV9-transfected cardiomyocytes diffusion from the injection site in the heart post-myocardial infarction in pigs. Transfected cells are to be identified by immunohistochemistry.

Specific Aim 2: To determine if cardiac function of pig hearts is improved with the post-MI injection of hydrogel-encapsulated virus carrying **well-established myocardial transcription factors.** ***The cardiac functional improvement is to be assessed by MRI.***

LITERATURE REVIEW

In pursuit of developing a more robust treatment for myocardial infarction (MI), the field of regenerative medicine seeks to replace or repair the heart after HF by direct reprogramming of cardiac fibroblast into functional myocardial cells. Cardiac fibroblasts are the most abundant cells that support the heart, and hence, a great source of initial cells for *in vivo* direct reprogramming. Ieda et al. in 2010 were the first to report induced cardiomyocytes (iCMs) from neonatal, adult cardiac fibroblasts, & mouse tail-tip dermal fibroblasts by the force expression of ectopic cardiac transcription factors, such as Gata4 (G), Mef2C (M), and Tbx5 (T) (a.k.a GMT) (Ieda et al., 2010). After this group, other scientists including John Gearhart's laboratory, have improved this technique by using different combinations of transcription factors, microRNAs, small molecules, and using human cardiac fibroblasts as the initial cell source (Inagawa et al., 2012; Jayawardena et al., 2012; Nam et al., 2013; Qian et al., 2012; Song et al., 2012). For example, Ifkovits et al., demonstrated that the addition of small molecules (i.e. TGFβ inhibitor) along with ectopic expression of Hand2, Gata4, NKX2.5, and Tbx5 (HNGMT) was sufficient to induce direct reprogramming of both mouse embryonic and adult fibroblasts into mature cardiomyocyte cells (Ifkovits, Addis, Epstein, & Gearhart, 2014). This work was validated by staining for cardiac-specific markers like α-actinin, α-myosin heavy chain, myosin light chain 2, and myosin light chain 7 and by the expression of endogenous cardiac markers. Ifkovits et al. work was recently validated by an independent group *in vitro* and *in vivo (*Mohamed *et al., 2017)*. These induced cardiomyocytes had strong synchronized beating and a similar gene expression pattern to endogenous cardiomyocyte cells. Yet whether this

reprogramming could occur in large mammals, and be transitioned into the clinic, remains unclear

Significant understanding of the basic biology of heart development has come from small animal models such as mice (Xin, Olson, & Bassel-Duby, 2013). This work is valuable because this is how the field of reprogramming knows which genes are critical in the formation and proper function of the heart. One serious limitation of this work is that the human and mouse heart differ in heart rate, size, and oxygen consumption. As a result, it is challenging to use mice as animal model to generate therapeutic treatment for MI. Thus, a large animal model that shares physiological and pathological similarity to the human heart is more applicable than mice (Dixon & Spinale, 2009). The ideal large animal model that is clinically relevant is the pig because it's heart resembles the human heart more closely in size, function, and anatomy. Hence, we are trying to see if technologies developed in mice can be translated into the human clinic using a porcine model

Preliminary work from our laboratory demonstrated that *in vitro* reprogramming of adult cardiac fibroblast into cardiomyocytes is possible, as shown in Figure 3. This figure show the used of different transcription factor to reprogram cardiac fibroblast from pig into induced cardiomyocytes. When similar work was performed *in vivo* the transfected cells were low in number and not found where the virus was injected. Despite the low efficiency of cell expression GFP and diffusion of the virus, this work revealed that adeno-associated virus serotype 9 (AAV9) was effective in forcing the expression of green fluorescent protein (GFP) in cardiomyocytes and fibroblasts (data not shown). Furthermore, this virus has been demonstrated to have the capacity to infect cardiomyocytes as demonstrated by

Inagawa K et al., 2012; thus, this virus was selected for the *in vivo* experiment. In order to improve our method of direct reprogramming *in vivo*, we decided to use hydrogel as a delivery system to improve the efficiency of gene delivery and retention at the infarct zone, which is the compromised area, and border zone, which is the histologically normal part of the heart.

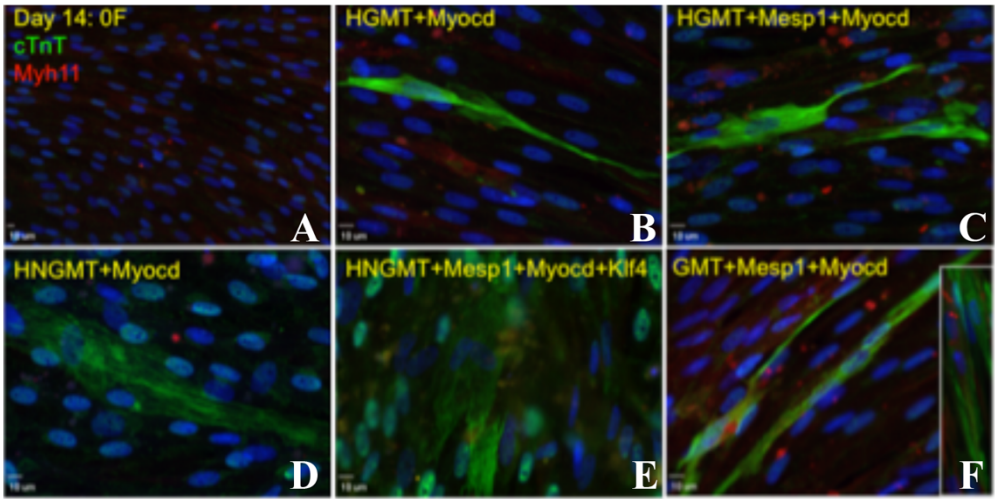

Figure 3. Adapted from unpublished data (Ifkovits 2014). Representative immunostaining of porcine iCMs at Day 14 *in vitro*. A) At day 14, staining of cTnT and Myh11 of a control sample of zero factor. B) Staining of cTnT of an experimental sample with the combination of HGMT + Myocd, C) addition of Mesp1, D) addition of NKX2.5, E) addition of KLf4, finally F) the combination of GMTMM. These result show early sarcomeric structures with the combination of different factors.

Hydrogels, both natural and synthetic, have been effectively explored as a delivery system of cells and protein *in vivo* and *in vitro* to enhance cardiac metabolic functions post-MI in mice and in pigs (Hasan et al., 2015) (Atluri et al., 2014; Gaffey et al., 2015). This delivery system has been also used for cartilage repair in a Mini-Pig model (Fisher et al., 2016). Hydrogels are an ideal candidate for many biomedical applications because they are biodegradable and form injectable three-dimensional polymeric networks that retain their integrity and property after self-assembly (Rodell et al., 2015). Since it has been established

in the field of bioengineering that hydrogels are an ideal candidate for delivery of protein, small molecules, and cells, we experimented in delivering AAV9-GFP and AAV9-Transcription Factors to pig hearts post-MI using hydrogels.

3. MATERIALS AND METHODS

3.1. Ethics Statement

All the experiments concerning animals were conducted under a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

3.1.2 Adeno-Associated Viral Vector 9 Construction.

Adeno-associated viral vector (AAV-9)-Cytomegalovirus (CMV)-Green Fluorescent Protein (GFP), red fluorescent protein mCherry, Myocyte Enhancer Factor 2C (MEF2C), T-Box Protein 5 (TBX5), Mesoderm Posterior BHLH Transcription Factor 1 (MESP1), and Myocardin (MYOCD).

3.1.3 Hydrogel Synthesis and Preparation

Cyclodextrin-modified HA (CD-HA), ~28% of repeat units modified, synthesis described elsewhere with reagents available from Sigma (St. Louis, MO) (Gaffey et al., 2015).

3.1. 4 Experimental Design

As shown in Fig. 5, the animals had an MRI at day 0 to assess their cardiac functional output and after this the infarct was induced, as explained later in the method section. Three days post-MI, all eleven animals underwent surgery for the injections. The infarct was first identified and then thin MRI compatible platinum wire was used to mark the ischemic area. Each injection was administered with an insulin syringe containing 200 μl of appropriate solution. Each animal received nine injections in the infarct and one on each side of the

compromised area. The injections were marked with platinum wire sutured on the epicardium (as shown in Fig. 5C).

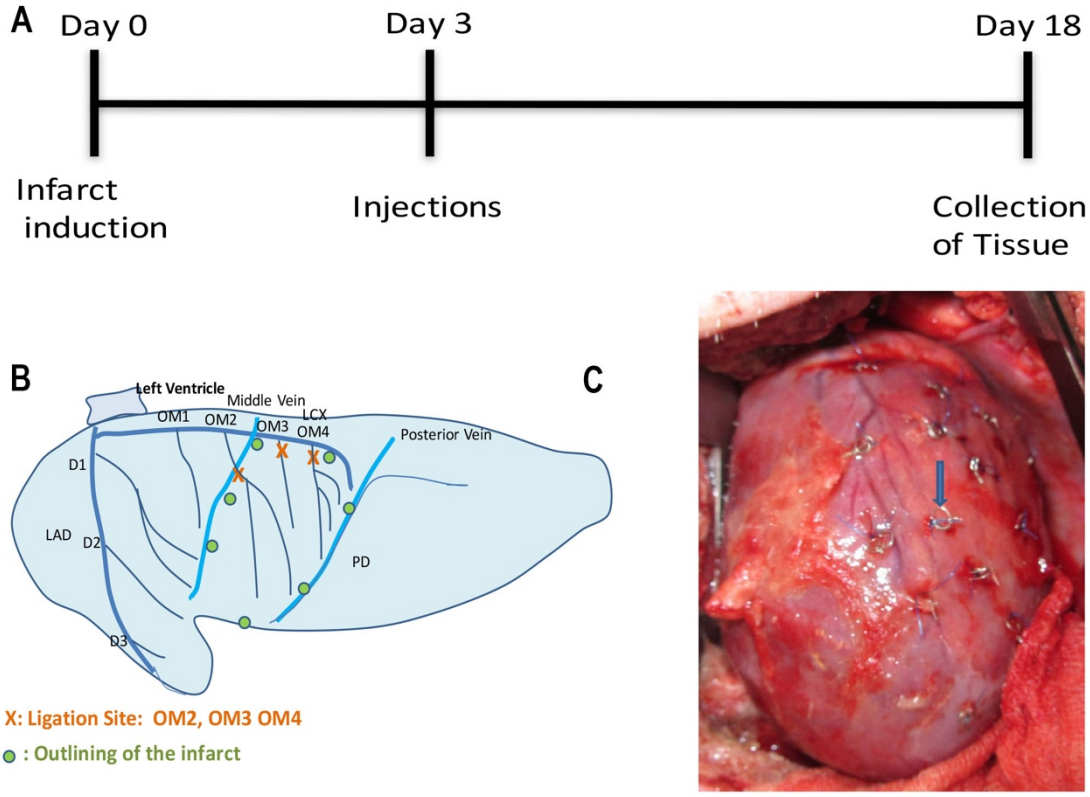

Figure 4. Timeline, procedure of infarction, and injections of Mep69. (A) Timeline of the experiment. (B) Generation of a posterolateral infarct comprising of 20% of the left ventricle by permanently ligating obtuse marginal arteries. (C) At day 3, the animal received eleven injections, shown with a blue arrow, labeled with compatible MRI platinum metal.

3.1.3 Porcine Infarct Model

Eleven male Yorkshire swine weighing 36-47 kg were used in this study. There were two groups: n=5 hydrogel/AAV9-TFs (experimental), n=6 saline (control). All the animals in this study had an MI. The animals were first anesthetized and sedated then, the LV free wall was exposed through a left thoracotomy. The animals were then sedated with

intramuscular ketamine injections (25-30 mg/kg), intubated, and mechanically ventilated. General anesthesia was maintained on mixed isoflurane (1.5-3.0%) and oxygen, which was delivered by volume-controlled ventilation (tidal volume 10-15 mg/kg) (Rodell et al., 2016). A posterolateral infarction was induced by the ligation of the left circumflex artery (LCX) and some obtuse marginal (OM) branches. Specific arteries were not targeted because pigs have some variations in their artery branches. Thus, to produce a reproducible infarct in all the animals, the OM branches of the posterolateral area in the LV were ligated. This area of the posterolateral LV is defined as the area between the middle coronary vein and posterior coronary vein, as shown in Fig 5 and Table 1 shows the variation of ligated arteries that supplied blood to the LV of the animals and in all cases, this pattern of coronary ligations produced an infarct comprising of approximately 20% of the left ventricle. For the first trial of this project, animal number 69 had an infarct produced as explained below. On day 0, three obtuse marginal (OM) branches (i.e., OM1 – OM3) were ligated producing a posterolateral infarct comprising of 20% of the left ventricle. Three days post-infarct, 100µl (total volume) of AAV9-GFP (AAV9. CMV. bi-nls. AsGFP. hGH. WPRE [$6.08x10^{13}$ GC/ml]) and HA-hydrogel were delivered to nine sites via an insulin syringe. These injections were in the infarcted region and one to each side of the border zone of the compromised area of the left ventricle.

3.1.4 MRI and Analysis

Image acquisition was performed at 3T (Tim Magnetom Trio Scanner; Siemens, Inc.). For longitudinal analysis of myocardial geometry and function *in vivo,* imagining was performed at baseline (immediately before infarct) as well as at 2 and 4 weeks post infarct.

Anesthesia was maintained throughout the procedure, and cardiac gating was performed by placement of a pressure catheter (Millar Instruments, Inc.) in the LV. Myocardial geometry was assessed from 2-dimensional CINE images, with additional late gadolinium enhancement imaging to confirm the infarct location (Rodell et al., 2016).

3.1.5 Histological Analysis of Pig Hearts 4 Weeks Post-MI

Four weeks post-MI, the animal underwent the final Magnetic resonance imaging (MRI), and the hearts were harvested for histological examination. The hearts were cut into seven slices; three infarcts regions, two borders, and two remotes, as shown in Fig. 6B. The injection sites were cut into approximately 4 cm blocks, rinsed with 1X Phosphate-buffer saline, (PBS) pH 7.4 (ThermoFisher Scientific, USA) and fixed in 4% paraformaldehyde at 4°C for 72 hours (Sigma-Aldrich, Germany). After fixation, the tissues were serially dehydrated in an ethanol series (75%, 95 %, and 100% of ethanol) for 48hr (ThermoFisher Scientific, USA). Finally, the blocks were cut down the middle to expose the myocardium wall and embedded in paraffin for serial sectioning (5-10 μm). To assess the infarcts, the tissues were stained with Hematoxylin and Eosin and Masson's Trichrome by the Cardiovascular Research Center (Histology and Gene Expression Core) at the University of Pennsylvania.

3.1.6 Immunohistochemistry Staining for Pig's Heart

Sectioned tissues were deparaffinized and rehydrated: xylene two times for 5 minutes (min), 50% xylene/ethanol for 5 more min, followed by 3 min incubations each in 100%, 95%, 75%, and 50% ethanol, and finally 3 min in tap water. Antigen retrieval was

performed with citrate buffer (97°C for 20 minutes followed by cooling to room temperature for 20 minutes) and rinsed with 0.1% PBS-Triton (PBST).

Slides were incubated with blocking buffer (e.g. 10% goat serum in 1% Bovine serum albumin (BSA)) for 1 hour at room temperature and the tissue was then incubated with the appropriate antibody mixture overnight at 4°C. The slides were rinsed for PBST 3 times for 10 min at room temperature followed by incubation with the appropriate secondary antibody for 1 hour at room temperature. The tissues were again rinsed with PBST 3 times for 10 min at room temperature. Then, incubated with 70% of ethanol for 5 min. The pig heart has a great amount of autofluorescence and therefore the tissues are incubated for 15 min at room temperature with an Autofluorescence Eliminator reagent (Millipore, USA). This reagent stains the tissues blue - and in order to remove this color and any Sudan black precipitate - the sample is rinsed multiple times with 70% of ethanol followed by a final wash with 1x PBS for 10 min and mounted with ProLong Gold antifade plus DAPI (ThermoFisher Scientific, USA). To examine the expression of the reporters (mCherry or GPF) or the V5-tagged transcription factors, the tissues were with stained with antibody against mCherry, GPF, or V5.

3.1.7 Image acquisition

Microscopic images were taken under fluorescence microscopy at 10X, 20X, and 40X magnification and assessed with Fiji (ImageJ) (Schindelin et al., 2012).

3.1.8 Statistical Analysis

GraphPad Prism 7 was used to compared the groups. Randomization was not applied.

Results

In the first part of this study, one pig (Mep69) was used to test the ability of hydrogel to improve transfection of cardiomyocytes with AAV9 expressing GFP (Fig. 5).

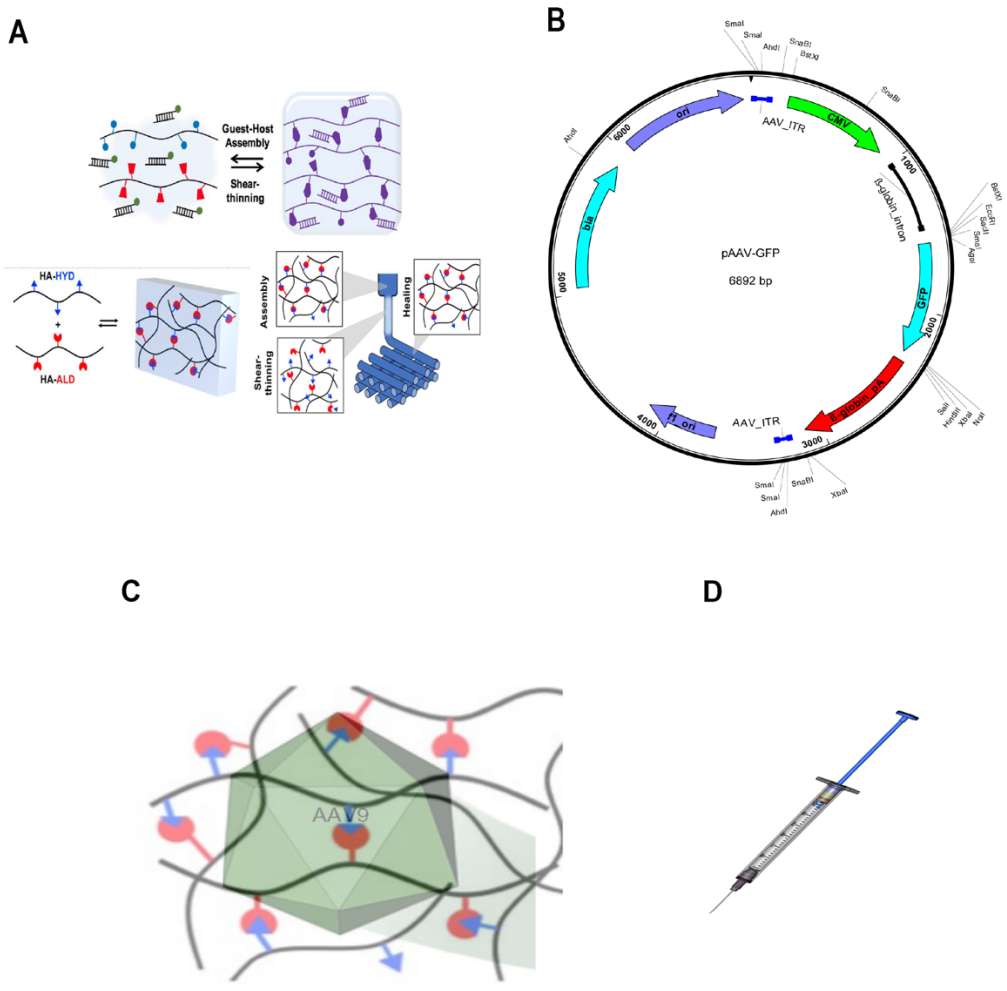

Figure. 5. A diagram of the Hydrogel, AAV-9.CMV.eGFP plasmid, the mixture of the virus and plasmid for injections used in the experiment. (A) Schematic of dynamic cross-link formation utilizing guess-host chemistry. (B) AAV-9.CMV.eGFP plasmid used in this experiment. (C) The mixture of the plasmid/AVV9 and hydrogel. (D) The mixture of virus and hydrogel was then used to inject the animal.

This animal had an induced infarct at day 0 and three days post-MI, eleven injections of AAV9-GFP encapsulated in hydrogel were given to the animal (Fig. 4). At day 18 post-MI, the animal was sacrificed and heart tissue collected for examination (Fig 6).

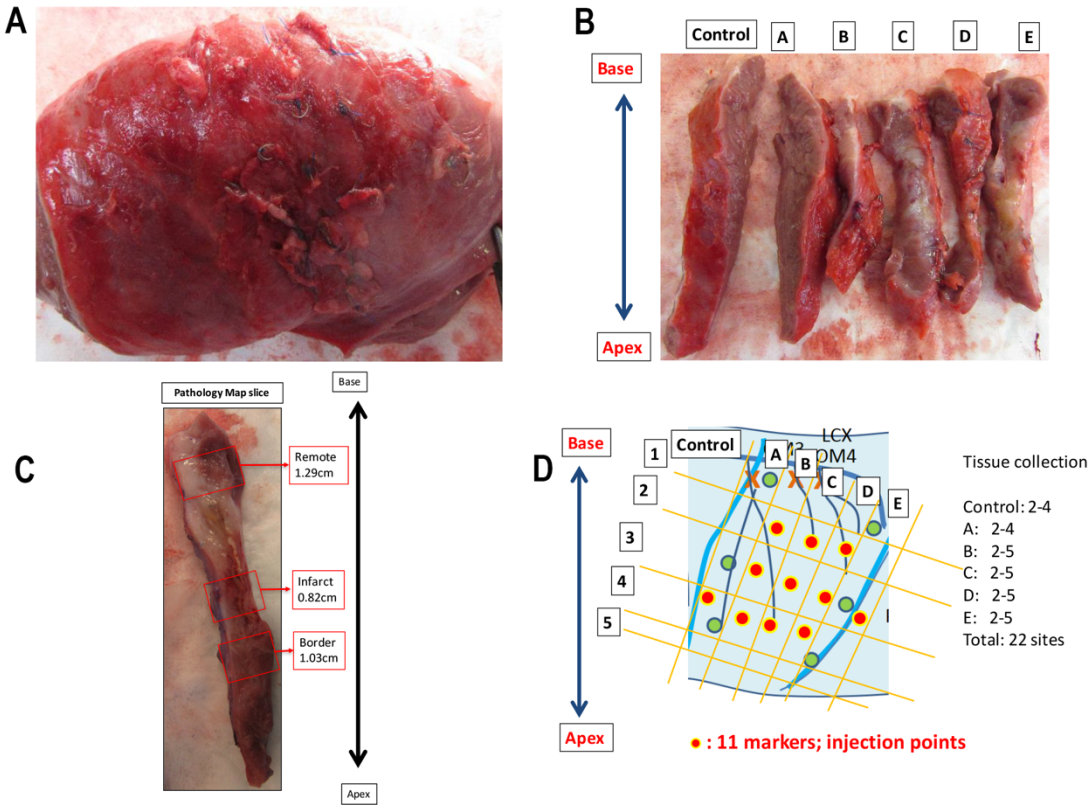

Figure 6. At day 18, the animal was sacrificed and the tissue was collected for analysis. (A) The heart was removed from the animal and evaluated to identify the infarct. (B) The infarcted and border zones were cut into slices. (C) The thickness of the remote, infarct, and border zone were measured.

Both the compromised and border zones (that is healthy surrounding tissue) were collected for immunohistochemistry and established markers of cardiomyocyte identity like cardiac Troponin were used to stain the tissues. The GFP-adenovirus transduced cells (GFP+) were

found within most of these two zones along with overlapping expression of GFP and cardiac Troponin T (cTnT or TnnT2), a cardiomyocyte marker (Figure 7).

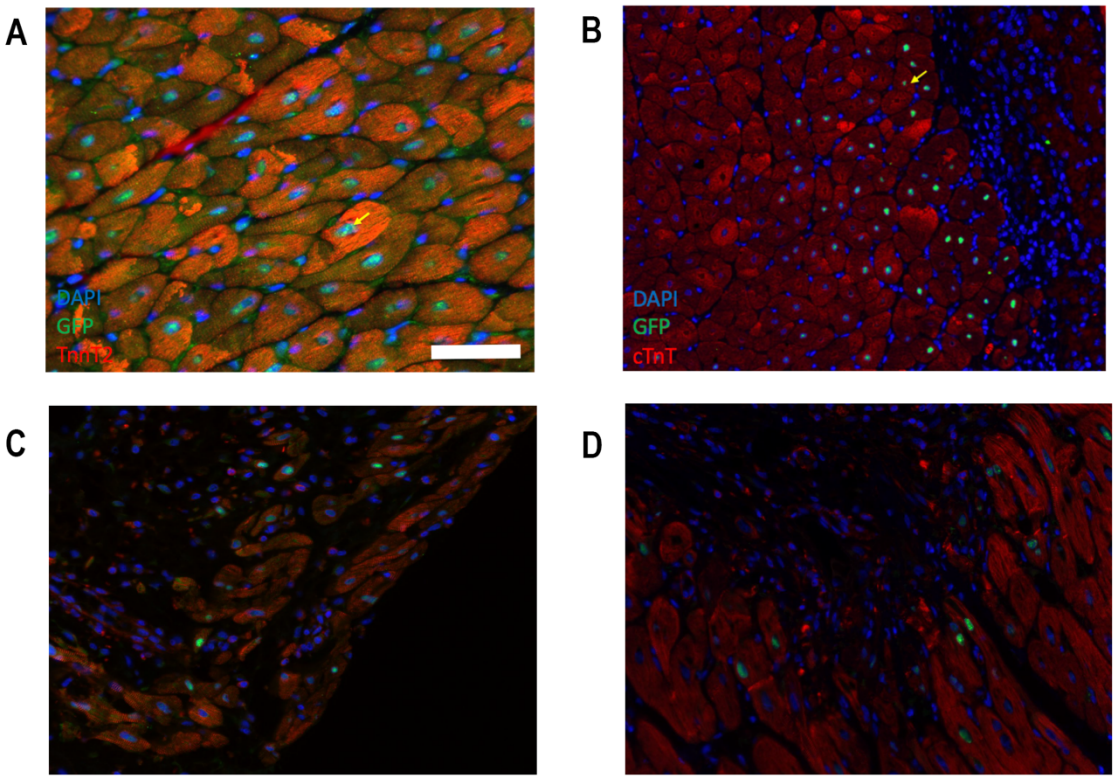

Figure 7. Immunofluorescence imaging of GFP^{+} cells, 18-days post-MI. Sections were mounted with DAPI (blue) and stained for GFP (green) and cTnT (red). Representative images of injection sites of the infarct and border zone. These are representative images of GFP+ cells at sites A, B, C and D of the infarct zone. Scale bar of 20μm. This figure demonstrates the expression of GFP in cardiomyocytes cells in the infarct and border zone.

GFP+ cells were immunopositive for cardiomyocytes and for vimentin (a fibroblast marker), and β-actin cells. However, there were no cells immunopositive for CD34 (a hematopoietic and endothelial marker), as shown in figure 8.

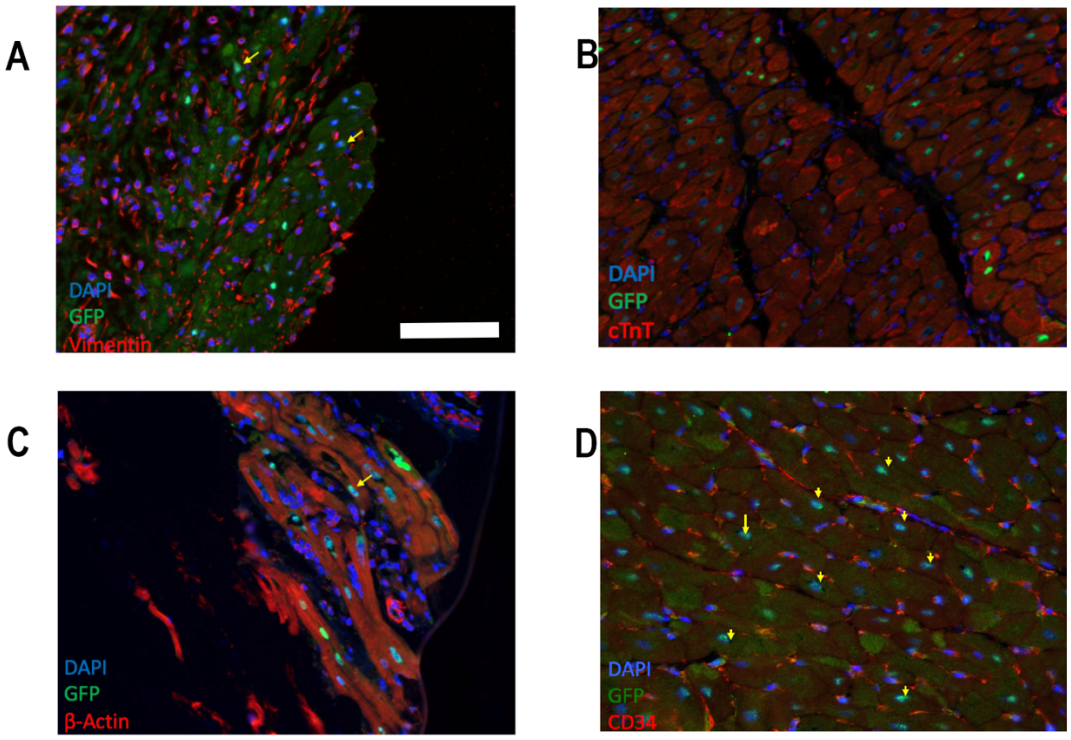

Figure 8. Co-staining of different markers to identify which cells expressed GFP. (A) Staining of vimentin (red), a fibroblast marker, (B) cTnT (red) stains for cardiomyocytes, (C) β-Actin (red) a smooth muscle cells marker, and (D) CD34 (red) an endothelial marker. Scale bar of 20μm. This image shows the expression of GFP mostly in cardiomyocytes and fibroblasts.

Hydrogel residue was not detected in the tissue used for analysis, which is consistent with a previous report of *in vivo* degradation (Dorsey et al, 2015).

This result stood in stark contrast to our earlier studies where few cells were found to be transduced when the GFP adenoviruses were used. In the pig Mep69, we could easily count the number of GFP-transduced cells within the hydrogel co-injected sites; but in our earlier studies there were very low number of positive cells. With the hydrogel, it was easier to find the location of the injection tract in the tissue making it easier to identify the

locations of the GFP+ cells in both the compromised area and the histologically normal areas. Our data strongly suggests that this injectable system increases virus retention during and after injections, with many GFP positive cells, demonstrating the efficiency of hydrogel as a potential therapeutic delivery system to treat HF.

Since the first experiment was very encouraging, we decided to continue with this experiment but with more animals to perform *in vivo* reprogramming. In order to test *in vivo* reprogramming in pigs, the combination of the following transcription factors (TFs) were used: Gata4, Mef2c, Tbx5, Mesp1, and Myocardin (GMTMM). This mixture of TFs was used to reprogram both mouse and human fibroblast cells into cardiomyocytes *in vitro* (Wada et al., 2013) and was validated by our laboratory.

In the second part of this project, we kept the animals alive for one more week and MRI analysis was performed before the infarct was induced, at two weeks post-MI, and at four weeks post-MI, just before the heart was harvested (Diagram 1). This time, there were two groups: n=5 hydrogel/AAV9-TFs (experimental), n=6 saline (control) (Table 1).

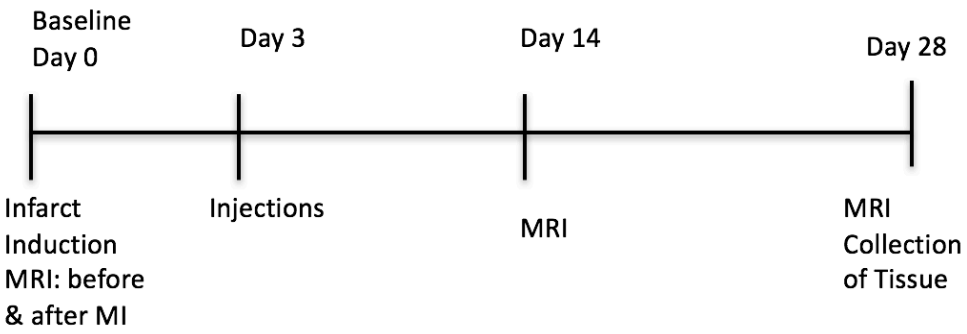

Diagram 1. Timeline of the second part of the experiment.

	Acute MI induction	Baseline					Infarct Thickness		
Animal	Ligation sites	Weigh	Infarct area	Injections	Infarct area	Infarct size	Border zone	Infarct	Remote
G,V&TF: 70	OM2,OM3 & OM4	36kg	~20%	11	17.92%	15.09cm²	1.03cm	0.82cm	1.29cm
G,V&TF: 71	OM2,OM3 & OM4	36kg	~20%	11	12.60%	10.09cm²	.85cm	0.70cm	1.02cm
G,V&TF: 72	D1, OM1, OM2,OM3 & OM4	38kg	~20%	11	16.13%	12.59cm²	0.80cm	0.55cm	0.90cm
G,V&TF: 74	OM2,OM3 & OM4	40kg	~20%	11	16.80%	15.32cm²	1.21cm	0.81cm	1.69cm
G,V&TF: 75	OM2,OM3*2,OM4,OM5 &OM6*2	47kg	~20%	11	14.80%	12.86cm²	1.02CM	0.91cm	0.95cm
Saline:82	OM1,OM2, OM3,OM4, &OM5	42kg	~20%	11	18.20%	15.31cm²	1.1cm	0.69cm	0.95cm
Saline:83	OM1,OM2, OM3,OM4, & branches of PD	41kg	~20%	11	17.30%	14.66cm²	0.94cm	0.64cm	1.33cm
Saline:84	OM2,OM3, OM4&OM5	40kg	~20%	11	18.60%	15.44cm²	0.95cm	0.71cm	1.32cm
Saline:85	OM1,OM2, OM4,OM5, &OM6	42kg	~20%	11	19.70%	17.87cm²	0.79cm	0.62cm	1.32cm
Saline:86	OM1,OM2, OM3,OM5, &OM6	42kg	~20%	11	19.50%	16.51cm²	0.91cm	0.58cm	1.23cm
Saline:87	OM3,OM4, &OM5	40kg	~20%	11	20%	15.48cm²	0.82cm	0.60cm	1.30cm

Table 1. Detailed records of how the infarct was induced, weight of animals, infarct sites, number of injections, and size of the compromise tissues. Abbreviations: G (hydrogel), V (AAV9), and TF (transcription factors), OM (obtuse marginal) and PD (posterior descending)

The number of injections, tissue collection, and analysis were the same as previously described for Mep69 (Fig. 5B & 5C and Fig. 6). Immunohistochemistry was also performed to detect the V5-tag expressed on each of the transcription factors in the same

manner as for Mep69. Cells immunopositive for V5 was strong, as shown in Fig. 9.

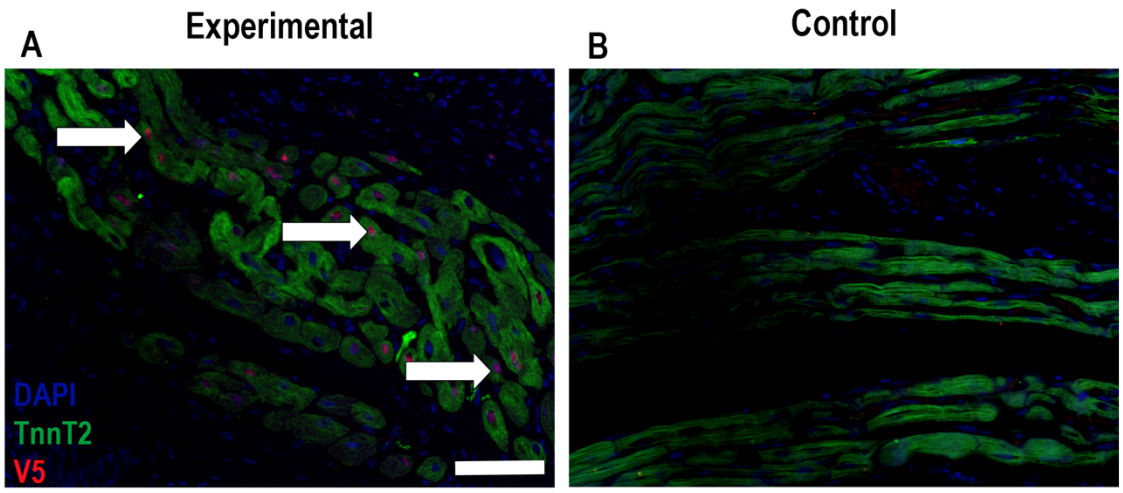

Figure 9. Representative images of immunohistochemistry of heart tissues in both experimental and control groups, 4 weeks post-MI. Sections were mounted with DAPI (blue) and stained for V5 (red) and TnnT2 (green). (A) and (B) are co-staining of TnnT2/V5 in the infarct zone. The white arrows on (A) points at V5+ cells in cardiomyocytes. Scale bar of 20μm. This image shows the expression of V5 in cardiomyocytes.

Moreover, in order to detect the formation of scar tissue or fibrosis, Masson's Trichrome staining (Fig. 10) was performed in both groups (i.e. experimental and control). According to this staining, there is cardiomyocytes in the infarct zone of the experimental animals.

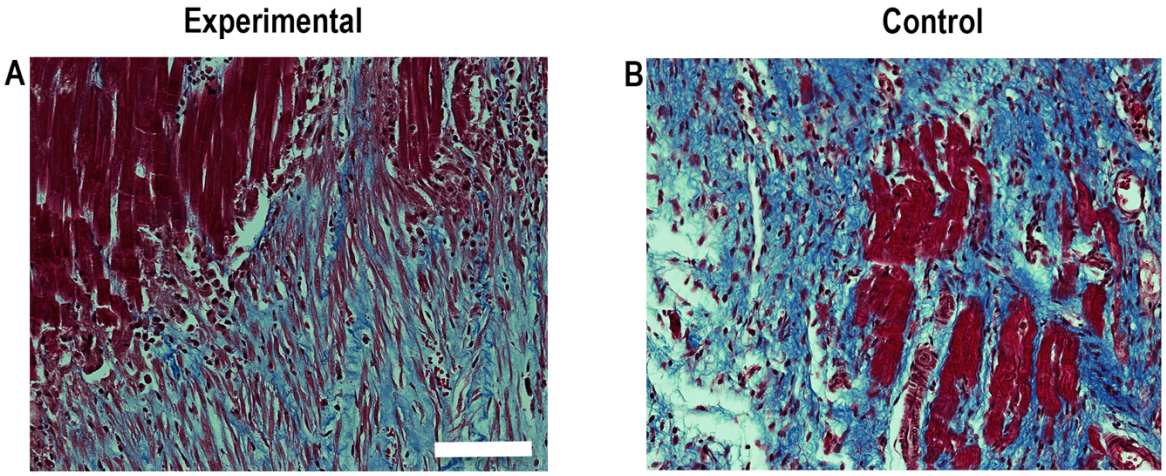

Figure 10. Representative images of trichrome staining. Trichrome staining of the infarct zone of experimental (A) and (B) group 4-weeks post-MI. Scale bar of 20μm.

The final analysis, clinically relevant to MI, was the assessment of cardiac function. A healthy human heart is one that has the ability to hold approximately 120ml of blood in the left ventricle before contraction; a process known as left ventricle end-diastolic volume (EDV). After the heart contracts, 50ml of blood is left in the ventricle; this ejection of blood into the aorta is called left ventricle end-systolic volume (ESV) and the measured difference of EDV-ESV is the ejection fraction (EF). After an MI, the heart forms scar tissues that reduces ventricular contractility, thereby affecting EDV, ESV, and EF.

Before the animals had the infarct, their left ventricle was functioning normally (Fig. 11, Baseline). However, after the infarct was induced, cardiac function was evaluated at two weeks and four weeks post-infarct, control (saline treated) animals had difficulty contracting their left ventricle (Fig. 11). In contrast, the experimental group appeared to have a small recovery of left ventriclular contractivity as ejection fraction increased by 4-

weeks post-MI. This result indicates that the use of these transcription factors, along with hydrogen, enable the left ventricle to regain contractility.

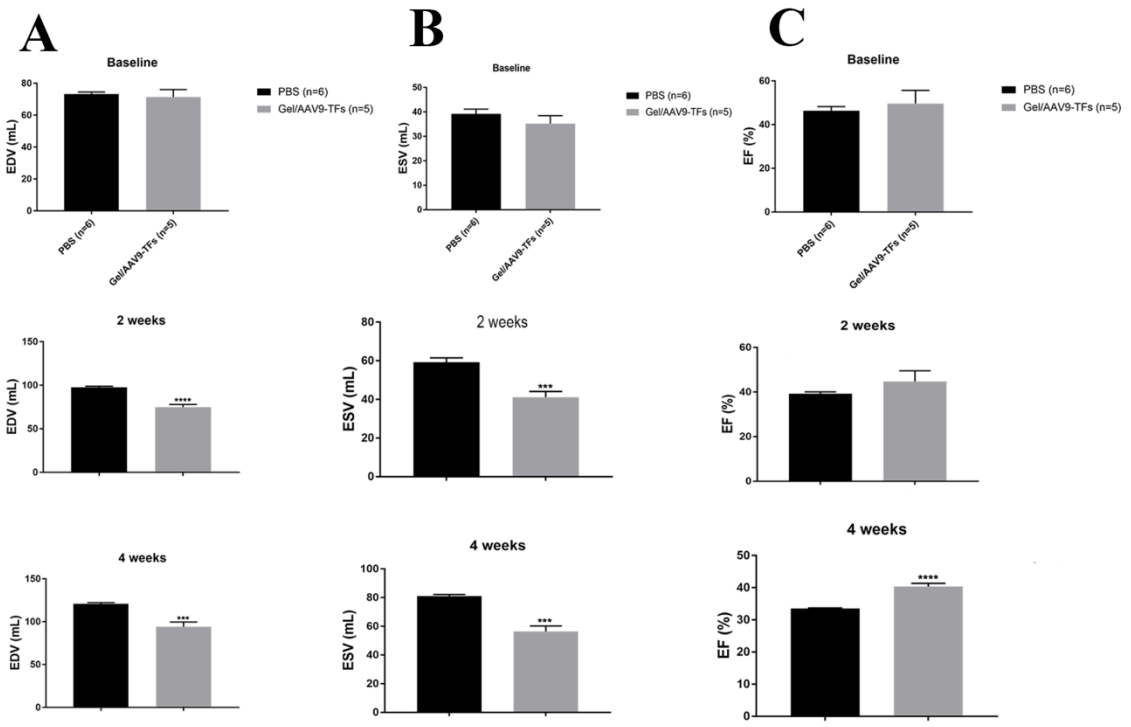

Figure 11. Magnetic resonance imaging (MRI) assessment of cardiac function. MRI measurements of left ventricular end-diastolic volume (EDV) (A), left ventricular end-systolic volume (ESV)(B), and ejection fraction (EF)(C). This figure demonstrates a functional cardiac volume improvement in the experimental animal compared to the control. P-values: EDV two weeks p <0.0001 ****, EDV four weeks P value 0.0007 ***, ESV two weeks p value 0.0009***, ESV 4 Weeks: P value 0.0001 ***, and EF 4 Weeks: P value <0.0001 ****.

Discussion

The field of cellular reprogramming, particularly the conversion of fibroblast cells into cardiomyocytes to treat MI, has come a long way (Doppler, Deutsch, Lange, & Krane, 2015). For example, it has taken the field almost a decade to determine which transcription factors, microRNAs and small molecules are necessary to generate induced cardiomyocytes, though the efficiency of induction remains very low, both *in vivo* and *in vitro*. This is a problem because an infarcted heart will likely require a large number of mature contractible cells to regain normal function. In order to address this issue, many laboratories have repeated published work of reprogramming to increase the efficiency of induced cardiomyocytes, but the results are unfortunately not reproducible. According to Doppler et al., 2015, one way to move the field forward, would be to conduct more studies to understand the epigenetics of this technology and improve viral delivery (as shown in figure 12).

Our goal is to reprogram cardiac fibroblasts into cardioyocytes because fibroblasts are the most abundant cells in the heart and are the cells recruited during healing process into the ischemic area of the heart to form scar tissue (Doppler et al., 2015; Prabhu & Frangogiannis, 2016). To our knowledge, this is the first study to attempt *in vivo* reprogramming in a large animal model, such as a pig, with the combination of GMTMM, post-MI. This study is also the first in furthering the field of reprogramming to develop a treatment for the pathological condition created by MI. Our results show that the biodegradable hyaluronic acid hydrogel is effective in delivering AAV9 at the point of injection (figure 9), and that the delivered combination of transcription factors improved

cardiac function in the experimental group compared with the control animals (figure 11). Although, we lack the data that could explain the mechanism that is driving this outcome, future experiments could test if the combination of these factors rescue compromised cardiac cells or differentiate endogenous fibroblasts into new cardiomyocytes of the left ventricle post-MI. The following discusses some experimental modifications for investigating the mechanisms.

First, the CMV promoter driving the TFs in the virus is not specific to fibroblast cells but drives TF expression in many other cell types (Fig. 8). However, this promoter proved successful *in vitro* in inducing the expression of GFP in pig cardiac fibroblast cells in contrast to a specific promoter for fibroblast cells, for example Vimentin (data not shown). The second limitation of this study is that all the factors are upstream of the same protein tag. This epitope tagged protein is called V5 and it enables the detections of the factors by immunochemical methods providing an indirect visualization of where the TFs are being expressed and translated. The caveat of tagging all the factors with the same tag, V5, is that factors cannot be distinguished from each other. (Fig 9). In the future, this can be fix by using different protein tags like mCherry and GFP.

Finally, these pigs are not transgenic and thus it is difficult to trace endogenous fibroblast cells that become novel cardiomyocytes with the forced expression of GMTMM post-MI, like in mice. Earlier reprogramming studies *in vivo* have been performed in transgenic mice harboring cardiac fibroblast-specific markers such as vimentin, Fibroblast-specific protein-1 or periostin, allowing one to genetically track the fate of endogenous cardiac fibroblasts *in vivo* and investigate if reprogramming is actually occurring in the

targeted cells. This type of study also reveals the lineage of the cells that were converted into cardiomyocytes. The creation of transgenic pigs is not impossible or unheard of in biomedical research (Aigner et al., 2010), but a challenge of using transgenic pigs is that the process is costly and time consuming.

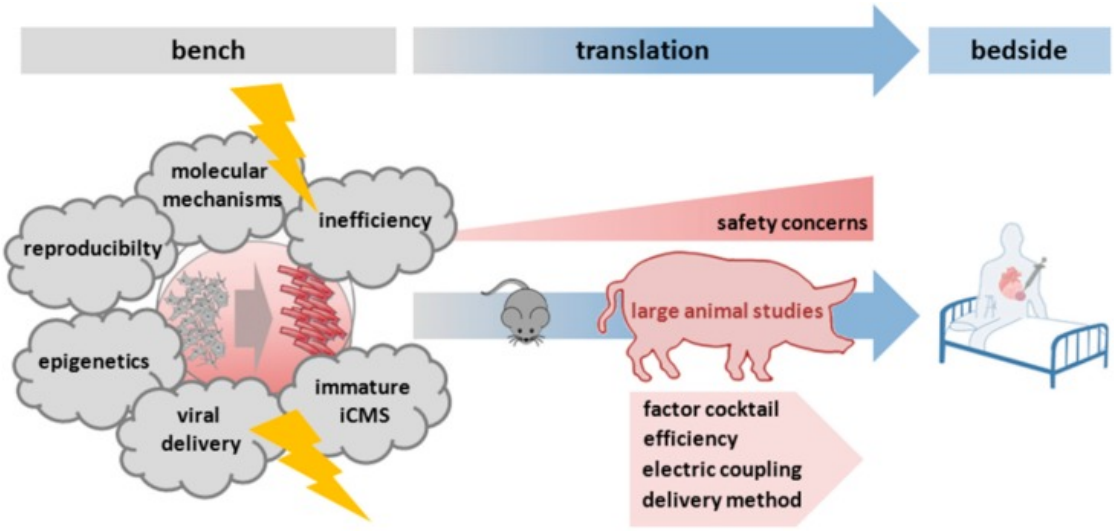

Figure 12. Image adapted from (Doppler et al., 2015). Before direct reprogramming is done in humans, the efficiency of induced cardiomyocytes needs to be increased. Further studies are necessary to understand the mechanism of this technology as this is a safety concern.

Conclusions

In summary, heart failure is an important health care issue because of its morbidity, cost of care, and mortality. Heart failure, which is one of the leading causes of death in the United States (Benjamin et al., 2017), is a consequence of Myocardial infarction (MI) also known as a heart attack due to the loss of blood flow and oxygen delivery to the cardiac tissue. If the MI is survived, the death of the hypoxic cardiac tissue results in a dysfunctional scar tissue that renders the individual more susceptible to further heart failure, hence, a treatment for this post-MI, pathological state is imperative. The current treatments of MI do not reverse the damage that the heart suffers from a heart attack and are incapable of making the heart generate new cardiomyocytes to replace the scar tissues that form after an MI. Reversing and redirecting the fate of mature cardiac fibroblasts into induced cardiomyocytes *in vivo* after an MI is an intriguing therapeutic modality. These cells have been extensively explored as an initial cell source for reprogramming into different kinds of cells.

In this study, we investigated the ability of a hyaluronic acid (HA) hydrogel to efficiently deliver a viral vector of transcription factors to post-MI pig hearts with the aim of increasing the number of functional cardiomyocytes in the damaged area of cardiac tissue. Our results suggest, a hydrogel was capable of delivering and retaining the viral vector at the sites of interest. This was shown by the staining of GFP+ cells and V5+ cells in the infarction. Moreover, our result also shows that using the combination of transcription factors Gata4, Mef2c, Tbx5, Mesp1, and Myocardin (GMTMM) carried in the hydrogel-embedded Adeno-Associated Virus (AVV9) leads to a modest recovery of

left ventrical ejection fraction as assessed by functional MRI imaging of the pig hearts. Our work is a proof-of-concept in the treatment of MI-damaged cardiac tissue.

In summary, these findings illustrate the value of using a combination of hydrogel and AVV9 for delivery of in vivo transcription factors as potential therapeutic treatment after MI. Intramyocardial injections of hydrogel and AVV9-TFs-V5 help reduce scar size of the MI, leading to an improvement in diastolic and systolic function. These findings have important clinical implications for future investigations of gene therapy for heart disease.

Appendix A: List of Software used

Fiji (ImageJ)

GraphPad Prism 7

Appendix B: List of statistical test

t-test

References

Aigner, B., Renner, S., Kessler, B., Klymiuk, N., Kurome, M., Wunsch, A., & Wolf, E. (2010). Transgenic pigs as models for translational biomedical research. *J Mol Med (Berl), 88*(7), 653-664. doi:10.1007/s00109-010-0610-9

Atluri, P., Miller, J. S., Emery, R. J., Hung, G., Trubelja, A., Cohen, J. E., . . . Woo, Y. J. (2014). Tissue-engineered, hydrogel-based endothelial progenitor cell therapy robustly revascularizes ischemic myocardium and preserves ventricular function. *J Thorac Cardiovasc Surg, 148*(3), 1090-1097; discussion 1097-1098. doi:10.1016/j.jtcvs.2014.06.038

Benjamin, E. J., Blaha, M. J., Chiuve, S. E., Cushman, M., Das, S. R., Deo, R., . . . Stroke Statistics, S. (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. *Circulation, 135*(10), e146-e603. doi:10.1161/CIR.0000000000000485

Boyle, A. J., Schulman, S. P., Hare, J. M., & Oettgen, P. (2006). Is stem cell therapy ready for patients? Stem Cell Therapy for Cardiac Repair. Ready for the Next Step. *Circulation, 114*(4), 339-352. doi:10.1161/CIRCULATIONAHA.105.590653

Chong, J. J., Yang, X., Don, C. W., Minami, E., Liu, Y. W., Weyers, J. J., . . . Murry, C. E. (2014). Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature, 510*(7504), 273-277. doi:10.1038/nature13233

Dixon, J. A., & Spinale, F. G. (2009). Large animal models of heart failure: a critical link in the translation of basic science to clinical practice. *Circ Heart Fail, 2*(3), 262-271. doi:10.1161/circheartfailure.108.814459

Doppler, S. A., Deutsch, M. A., Lange, R., & Krane, M. (2015). Direct Reprogramming-The Future of Cardiac Regeneration? *Int J Mol Sci, 16*(8), 17368-17393. doi:10.3390/ijms160817368

Dunlay, S. M., Shah, N. D., Shi, Q., Morlan, B., VanHouten, H., Long, K. H., & Roger, V. L. (2011). Lifetime costs of medical care after heart failure diagnosis. *Circ Cardiovasc Qual Outcomes, 4*(1), 68-75. doi:10.1161/CIRCOUTCOMES.110.957225

Fisher, M. B., Belkin, N. S., Milby, A. H., Henning, E. A., Soegaard, N., Kim, M., . . . Mauck, R. L. (2016). Effects of Mesenchymal Stem Cell and Growth Factor Delivery on Cartilage Repair in a Mini-Pig Model. *Cartilage, 7*(2), 174-184. doi:10.1177/1947603515623030

Gaffey, A. C., Chen, M. H., Venkataraman, C. M., Trubelja, A., Rodell, C. B., Dinh, P. V., . . . Atluri, P. (2015). Injectable shear-thinning hydrogels used to deliver endothelial progenitor cells, enhance cell engraftment, and improve ischemic myocardium. *J Thorac Cardiovasc Surg, 150*(5), 1268-1276. doi:10.1016/j.jtcvs.2015.07.035

Hasan, A., Khattab, A., Islam, M. A., Hweij, K. A., Zeitouny, J., Waters, R., . . . Paul, A. (2015). Injectable Hydrogels for Cardiac Tissue Repair after Myocardial Infarction. *Adv Sci (Weinh), 2*(11), 1500122. doi:10.1002/advs.201500122

Heidenreich, P. A., Albert, N. M., Allen, L. A., Bluemke, D. A., Butler, J., Fonarow, G. C., . . . Stroke, C. (2013). Forecasting the impact of heart failure in the United States: a policy statement from the American Heart Association. *Circ Heart Fail, 6*(3), 606-619. doi:10.1161/HHF.0b013e318291329a

Ieda, M., Fu, J. D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B. G., & Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. *Cell, 142*(3), 375-386. doi:10.1016/j.cell.2010.07.002

Ifkovits, J. L., Addis, R. C., Epstein, J. A., & Gearhart, J. D. (2014). Inhibition of TGFbeta signaling increases direct conversion of fibroblasts to induced cardiomyocytes. *PLoS One, 9*(2), e89678. doi:10.1371/journal.pone.0089678

Inagawa, K., Miyamoto, K., Yamakawa, H., Muraoka, N., Sadahiro, T., Umei, T., . . . Ieda, M. (2012). Induction of cardiomyocyte-like cells in infarct hearts by gene transfer of Gata4, Mef2c, and Tbx5. *Circ Res, 111*(9), 1147-1156. doi:10.1161/circresaha.112.271148

Jayawardena, T. M., Egemnazarov, B., Finch, E. A., Zhang, L., Payne, J. A., Pandya, K., . . . Dzau, V. J. (2012). MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. *Circ Res, 110*(11), 1465-1473. doi:10.1161/circresaha.112.269035

Laflamme, M. A., & Murry, C. E. (2011). Heart regeneration. *Nature, 473*(7347), 326-335. doi:10.1038/nature10147

Lemond, L., & Allen, L. A. (2011). Palliative care and hospice in advanced heart failure. *Prog Cardiovasc Dis, 54*(2), 168-178. doi:10.1016/j.pcad.2011.03.012

Li, M., & Izpisua Belmonte, J. C. (2016). Mending a Faltering Heart. *Circ Res, 118*(2), 344-351. doi:10.1161/CIRCRESAHA.115.306820

Mohamed, T. M., Stone, N. R., Berry, E. C., Radzinsky, E., Huang, Y., Pratt, K., . . . Srivastava, D. (2017). Chemical Enhancement of In Vitro and In Vivo Direct Cardiac Reprogramming. *Circulation, 135*(10), 978-995. doi:10.1161/CIRCULATIONAHA.116.024692

Murry, C. E., Chong, J. J., & Laflamme, M. A. (2014). Letter by Murry et al regarding article, "Embryonic stem cell-derived cardiac myocytes are not ready for human trials". *Circ Res, 115*(10), e28-29. doi:10.1161/CIRCRESAHA.114.305042

Nam, Y. J., Song, K., Luo, X., Daniel, E., Lambeth, K., West, K., . . . Olson, E. N. (2013). Reprogramming of human fibroblasts toward a cardiac fate. *Proc Natl Acad Sci U S A, 110*(14), 5588-5593. doi:10.1073/pnas.1301019110

Prabhu, S. D., & Frangogiannis, N. G. (2016). The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circ Res, 119*(1), 91-112. doi:10.1161/CIRCRESAHA.116.303577

Qian, L., Huang, Y., Spencer, C. I., Foley, A., Vedantham, V., Liu, L., . . . Srivastava, D. (2012). In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. *Nature, 485*(7400), 593-598. doi:http://www.nature.com/nature/journal/v485/n7400/abs/nature11044.html - supplementary-information

Rodell, C. B., Lee, M. E., Wang, H., Takebayashi, S., Takayama, T., Kawamura, T., . . . Gorman, R. C. (2016). Injectable Shear-Thinning Hydrogels for Minimally Invasive Delivery to Infarcted Myocardium to Limit Left Ventricular Remodeling. *Circ Cardiovasc Interv, 9*(10). doi:10.1161/CIRCINTERVENTIONS.116.004058

Rodell, C. B., MacArthur, J. W., Dorsey, S. M., Wade, R. J., Wang, L. L., Woo, Y. J., & Burdick, J. A. (2015). Shear-Thinning Supramolecular Hydrogels with Secondary Autonomous Covalent Crosslinking to Modulate Viscoelastic Properties In Vivo. *Adv Funct Mater, 25*(4), 636-644. doi:10.1002/adfm.201403550

Rojas, S. V., Kensah, G., Rotaermel, A., Baraki, H., Kutschka, I., Zweigerdt, R., . . . Martens, A. (2017). Transplantation of purified iPSC-derived cardiomyocytes in myocardial infarction. *PLoS One, 12*(5), e0173222. doi:10.1371/journal.pone.0173222

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., . . . Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat Methods, 9*(7), 676-682. doi:10.1038/nmeth.2019

Scudellari, M. (2016). How iPS cells changed the world. *Nature, 534*(7607), 310-312. doi:10.1038/534310a

Song, K., Nam, Y. J., Luo, X., Qi, X., Tan, W., Huang, G. N., . . . Olson, E. N. (2012). Heart repair by reprogramming non-myocytes with cardiac transcription factors. *Nature, 485*(7400), 599-604. doi:10.1038/nature11139

Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell, 126*(4), 663-676. doi:10.1016/j.cell.2006.07.024

Wada, R., Muraoka, N., Inagawa, K., Yamakawa, H., Miyamoto, K., Sadahiro, T., . . . Ieda, M. (2013). Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. *Proc Natl Acad Sci U S A, 110*(31), 12667-12672. doi:10.1073/pnas.1304053110

Xin, M., Olson, E. N., & Bassel-Duby, R. (2013). Mending broken hearts: cardiac development as a basis for adult heart regeneration and repair. *Nat Rev Mol Cell Biol, 14*(8), 529-541. doi:10.1038/nrm3619